Claims 1, 3-6, 9-14, 16-20, and 27-30 were rejected under 35 U.S.C. 103(a) as being obvious over Wooley by itself or in combination with Martin.

The Claimed Invention

The claimed invention includes vesicles made from triblock amphiphilic ABA copolymers, where one of A or B is hydrophilic and the other is hydrophobic, which self-assemble when dispersed in oil or water. The vesicles are hollow. Vesicles are defined on page 4 as "spontaneously forming aggregates having a generally spherical shape and an interior void." The resulting vesicles will have hydrophobic and hydrophilic layers arranged depending on the type of copolymer used.

The claimed invention further includes nanocapsules formed by stabilizing the vesicles made from ABA copolymers. The nanocapsules are also hollow. Stabilization can be through crosslinking of the copolymers, such as crosslinking of end groups of the copolymers.

The claimed invention further includes nanocapsules formed by end-group stabilization of amphiphilic copolymers. The copolymers do not have to be triblock copolymers.

Active agents can be encapsulated within the vesicles and the nanocapsules and targeting molecules can be attached to the vesicles and nanocapsules.

Analysis

112 Rejections

The term "hollow"

The Examiner argues that the term "hollow" as used in the claims is unsupported by the specification. In fact, the term is supported and is at the core of the invention as claimed. The term as added to the claims is redundant but was added to even more clearly emphasize the differences with the prior art to the Examiner. The term "vesicle" is clearly defined in the specification on page 4 as "spontaneously forming aggregates having a generally spherical shape and an interior void." An interior void means that the vesicle is hollow. The term "hollow" is defined on the online Merriam Webster dictionary (see Exhibit A) as "having a cavity within", such as a "hollow tree". Even though the interior of the tree is filled with air, it is still considered to be hollow. The

vesicles of the invention do not have polymer in the interior, thus they have an interior void, i.e. they are hollow (see Exhibit B for the definition of the term "void" from the online Merriam Webster dictionary). They most likely are filled with fluid if they are in a liquid environment but they are still "hollow" as that term is commonly used.

The term "molecule"

The Examiner maintains the rejection of claim 17 as indefinite due to use of the term "molecule". Claim 17 is directed to incorporation of a molecule into the vesicle membrane.

As supported by Exhibit C, the common definition of the term molecule is that it is the smallest particle of a compound (or protein, for example) that has the chemical properties of that compound. In fact, "one specific membrane protein" is not made of several molecules as stated by the Examiner. One membrane protein is a single molecule. This use of the term "molecule" is supported in depth in the specification on pages 19-21.

102 Rejections

Wooley

This rejection is again traversed because the reference does not teach or suggest hollow vesicles- i.e. vesicles having a shell enclosing an interior void. Wooley teaches microparticles formed from amphiphilic copolymers, having a crosslinked shell and an interior core domain. The hydrophilic portion of the amphiphilic copolymer forms the shell domain and the hydrophobic portion forms the interior core domain, or vice versa.

Micelles are commonly formed from amphiphilic molecules, having a hydrophilic (or hydrophobic) head region and a hydrophobic (or hydrophilic) tail region. The amphiphilic molecules assemble into spherical structures wherein the heads are on the periphery of the micelle and the tails are clustered in the interior. The interior is not a void- micelles are not hollow.

Methods of making the nanoparticles are described beginning on page 69. One method involves assembling the amphiphilic copolymers into a micellar structure and then crosslinking the outer hydrophilic or hydrophobic heads. As discussed above, micelles are not hollow- the tail regions are clustered in the interior. These tail regions can be crosslinked together, in one embodiment.

As further evidence that the nanoparticles taught by Wooley are not hollow, see Exhibit D and E, two articles from the Record, a school newspaper of Washington University in St. Louis school newspaper. Exhibit D, dated May 4, 2000 discusses "knedel" nanoparticles and states that Dr. Wooley had "recently announced" that she had "successfully hollowed out the knedel core to produce 'nanocages' ..." Exhibit E, dated September 14, 2001, states that "In 2000, Wooley and researchers in her lab hollowed out the knedel core to produce 'nanocages'...". These references show that in 1996, when the priority document for WO 97/49387 was filed, the "knedels" were not hollow.

Exhibit F is provided to show that the "knedels" referenced in the articles of Exhibits D and E are the particles disclosed in WO 97/49387. Under the "Materials and Methods" section in Exhibit F, it is stated that the experimental details for the preparation of the SCK (shell crosslinked knedel) structure are provided in an article published in 1997 (reference 21), and the description provided in Exhibit F corresponds to the structure described in Example 3 of WO 97/49387.

The Examiner's Arguments

The Examiner points to two sections of the reference for support that the particles of the reference are hollow- page 72, lines 19-22 and the paragraph linking pages 85 and 86. On page 72, the reference states "In preparing particles of the present invention, crosslinking of the shell domain, the interior core domain, or both, can be achieved ...." This simply means that the polymer making up the core domain may, or may not be, crosslinked. It does not mean that the "interior core domain" does not exist- i.e. that the core is empty (hollow). This does not imply that the core is not "solid"- it simply means that the core domain can be non-crosslinked material.

The Examiner also points to the paragraph spanning pages 85 and 86 which states

> The pharmaceutically active agent can be present in the particle dissolved in the interior core domain, or covalently attached to a component of the interior core domain, in the form of a fine dispersion within the interior core domain, or on the surface of the interior core domain, or at the interface between the crosslinked shell domain and the interior core domain.

The term "dissolved" does not mean that the interior domain is a liquid. See the paragraph directly above the one cited by the Examiner which states that the pharmaceutically active agent can be "dissolved in the crosslinked shell domain". Surely the Examiner will not argue that the crosslinked shell domain is also a liquid. The term dissolved is used in the same way in the paragraphs- to mean that the agent is present in the polymeric domain.

Pluyter

The rejection of the claims over Pluyter is again traversed. The present claims are drawn to vesicles and nanocapsules comprising membranes that are formed from amphiphilic copolymers. Pluyter teaches lamellar vesicles which may have copolymers "partially incorporated" therein (col. 5, lines 51-55). The copolymers may be attached to the vesicles or incorporated within the vesicles but they do not form the vesicle. In fact, the polymers make up only 0.1 - 10%, most preferably only 0.5 - 2% of the compositions (col. 3, lines 57-59). Applicants do not need to limit their claims to state that the membranes are "totally made from the copolymers" as suggested by the Examiner. It is clear that the claimed invention differs substantially from the cited art in that the membranes are "formed from amphiphilic copolymers". The claimed compositions, comprising lamellar vesicles having, at most 10% block copolymers, can not be said to be "formed from" the copolymers.

Martin

The rejection of the claims over Martin is also traversed. Martin teaches liposomes formed from lipids. The liposomes have block copolymers attached thereto. The membranes are not "formed from amphiphilic copolymers", as recited in the present claims. Again, Applicants do not need to limit the present claims to recite that they do not exclude liposomes and other material from contributing to the membrane, as suggested by the Examiner.

103 Rejection

Martin does not teach stabilizing vesicles to form nanocapsules. Wooley does not teach hollow vesicles. Accordingly, these references, taken alone or in combination, do not anticipate nor render the claimed invention obvious. This rejection is traversed.

Conclusion

None of the references that were cited teach or suggest hollow vesicles or nanocapsules formed from triblock amphiphilic copolymers. None of the references teach or suggest nanocapsules formed by end group crosslinking vesicles formed from amphiphilic copolymers. Accordingly, it is respectfully submitted that the references are not appropriate as the basis of rejection of the claims.

Respectfully submitted,

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Collen A. Beard

Date: July 28, 2003

Pending Claims

1. (Previously Amended) Hollow vesicles comprising membranes formed from amphiphilic copolymers having hydrophobic and hydrophilic segments, wherein the copolymers are ABA copolymers, and wherein one of A and B is hydrophobic and the other is hydrophilic.

3. (Previously Amended) Hollow nanocapsules formed by stabilization of the vesicles of claim 1.

4. (Previously Amended) Hollow nanocapsules formed by stabilization of vesicles comprising membranes formed from amphiphilic copolymers having hydrophobic and hydrophilic segments, wherein the vesicles are stabilized by end group polymerization of the copolymers.

5. The nanocapsules of claim 3, wherein the vesicles are stabilized via crosslinking of the copolymers.

6. The nanocapsules of claim 4, wherein the copolymers are AB copolymers, wherein one of A and B is hydrophobic and the other is hydrophilic.

9. The nanocapsules of claim 4, wherein an active agent is encapsulated within the nanocapsule.

10. The vesicles of claim 1, wherein an active agent is encapsulated within the vesicle.

11. The nanocapsules of claim 3, wherein an active agent is encapsulated within the nanocapsule.

12. The vesicles of claim 1, wherein the vesicles comprise a hydrophilic inner layer, a hydrophobic middle layer and a hydrophilic outer layer.

13. The vesicles of claim 1, wherein the vesicles comprise a hydrophobic inner layer, a hydrophilic middle layer and a hydrophobic outer layer.

14. The vesicles of claim 1, wherein the copolymers are U-shaped and the vesicles have a hydrophobic inner layer and a hydrophilic outer layer, or a hydrophilic inner layer and a hydrophobic outer layer.

16. The nanocapsules of claim 4, wherein the polymerization is via photopolymerization.

17. (Previously Amended) The vesicles of claim 1, wherein one or more molecules are incorporated into the vesicle membrane.

18. The nanocapsules of claim 3, wherein the hollow morphology of the nanocapsules is preserved when the nanocapsules are dry.

19. The vesicles of claim 1, wherein the vesicles are biodegradable.

20. The nanocapsules of claim 3, wherein the nanocapsules are biodegradable.

27. The vesicles of claim 1 further comprising targeting molecules bound to the surface of the vesicles.

28. The vesicles of claim 27 wherein the targeting molecules are selected from the group consisting of carbohydrates, proteins, folic acid, peptides, peptoids, and antibodies.

29. The nanocapsules of claim 4, wherein the hollow morphology of the nanocapsules is preserved when the nanocapsules are dry.

30. The nanocapsules of claim 4, wherein the nanocapsules are biodegradable.

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hollow[1,noun]
hollow[2,adjective]
hollow[3,verb]
hollow[4,adverb]
Go

Main Entry: **[2]hol low**
Pronunciation: 'hä-(")lO
Function: *adjective*
Inflected Form(s): **hol low er** /'hä-l&-w&r/; **hol low est** /-l&-w&st/
Etymology: Middle English *holw, holh,* from *holh* hole, den, from Old English *holh* hole, hollow -- more at HOLE
Date: 13th century
1 : having an indentation or inward curve : CONCAVE, SUNKEN
2 : having a cavity within <*hollow* tree>
3 : lacking in real value, sincerity, or substance : FALSE, MEANINGLESS <*hollow* promises> <a victory over a weakling is *hollow* and without triumph -- Ernest Beaglehole>
4 : reverberating like a sound made in or by beating on a large empty enclosure : MUFFLED

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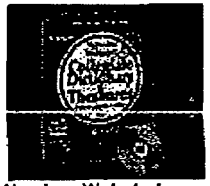

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void[1,adjective)
void[2,noun]
void[3,verb]
null and void

Main Entry: **2void**
Function: *noun*
Date: 1616
1 a : OPENING, GAP **b** : empty space : EMPTINESS, VACUUM
2 : the quality or state of being without something : LACK, ABSENCE
3 : a feeling of want or hollowness
4 : absence of cards of a particular suit in a hand orig. dealt to a player

molecule

A molecule is the smallest particle in a chemical element or compound that has the chemical properties of that element or compound. Molecules are made up of atoms that are held together by chemical bonds. These bonds form as a result of the sharing or exchange of electrons among atoms.

The atoms of certain elements readily bond with other atoms to form molecules. Examples of such elements are oxygen and chlorine. The atoms of some elements do not easily bond with other atoms. Examples are neon and argon.

Molecules can vary greatly in size and complexity. The element helium is a one-atom molecule. Some molecules consist of two atoms of the same element. For example, O_2 is the oxygen molecule most commonly found in the earth's atmosphere; it has two atoms of oxygen. However, under certain circumstances, oxygen atoms bond into triplets (O_3), forming a molecule known as ozone. Other familiar molecules include water, consisting of two hydrogen atoms and one oxygen atom (H_2O), carbon dioxide, consisting of one carbon atom bonded to two oxygen atoms (CO_2), and sulfuric acid, consisting of two hydrogen atoms, one sulfur atom, and four oxygen atoms (H_2SO_4).

Some molecules, notably certain proteins, contain hundreds or even thousands of atoms that join together in chains that can attain considerable lengths. Liquids containing such molecules sometimes behave strangely. For example, a liquid may continue to flow out of a flask from which some of it has been poured, even after the flask is returned to an upright position.

Molecules are always in motion. In solids and liquids, they are packed tightly together. In a solid, the motion of the molecules can be likened to rapid vibration. In a liquid, the molecules can move freely among each other, in a sort of slithering fashion. In a gas, the density of molecules is generally less than in a liquid or solid of the same chemical compound, and they move even more freely than in a liquid. For a specific compound in a given state (solid, liquid, or gas), the speed of molecular motion increases as the absolute temperature increases.

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Last updated on: Oct 15, 2002

Nanocages

Particles might provide new way to deliver gene therapy

By Tony Fitzpatrick

Wooley: Making strides with knedels

Chemists at Washington University have created tiny synthetic polymer particles that mimic viruses and show potential for a new direction in gene therapy and other biomedical applications.

The "nano" particle (a nano-meter is roughly one-billionth of a yard) has the unlikely name of "knedel" (k-ned-l) because of its similarity to a popular Polish dumpling filled either with meat or sweets. The knedels are shell cross-linked structures surrounding a hydrophobic -- water insoluble -- core domain. They have diameters ranging from 10 to 100 nanometers, so that they are of similar size to many globular proteins and viruses. In the body, they are expected to escape detection by the immune system.

Karen L. Wooley, Ph.D., professor of chemistry in Arts & Sciences, recently announced that she and her colleagues, Jianquan Liu, Ph.D., and Qi Zhang, Ph.D., both research assistants in chemistry, and Tomasz Kowaleski, Ph.D., research assistant professor in chemistry, have successfully hollowed out the knedel core to produce "nanocages" and attached a fluorescent tag to the core. They also attached a polypeptide called protein transduction domain (PTD) to the exterior of the nanostructure. They got this idea from Steven F. Dowdy, Ph.D., assistant professor of pathology at the Washington University School of Medicine, who demonstrated the PTD's efficiency in transducing proteins into cells.

With the aid of extremely powerful microscopes, Wooley and her colleagues were able to detect the peptide-bearing knedels binding to cell surfaces. Another group of nanoparticles without the PTD but with the fluorescent tags did not bind to target cells.

The accomplishment is a step toward using the knedel nano-particles as potential gene therapy carriers, or vectors. Most gene therapy attempts today use live viruses that are weakened to carry RNA, DNA or other therapeutic payloads. However, gene therapy has met with great difficulties since its inception a decade ago, and much of the trouble surrounds the safe use of live viruses. The difficulty reached tragic proportions in September 1999, when an 18-year-old gene therapy patient died after

being injected with a genetically altered adenovirus carrying a gene to control the youth's enzyme deficiency.

Wooley's knedels are biomimics -- they are designed to behave like viruses, which biochemically are attracted to hosts that they seek to infect. But a biomimic does not run the risk of a live virus, which, as in the case of the 18-year-old who died, might have toxic effects.

"We're combining synthetic constructs with biological pieces," Wooley explained. "It's what is called bioconjugation, and it's really a whole new territory for us. We're interested in making nanoparticles with the hollow cages into which one could put peptides, genes, proteins and small molecule drugs, all sorts of biomedical possibilities, even scavenging other cells or molecules. Other researchers are doing similar things in particle research, but they can't seem to get down to the same size range that we can."

Wooley presented the details of these latest results at the American Chemical Society's National Meeting, held this spring in San Francisco.

Beyond gene therapy, Wooley and her group intend to explore the potential of the knedels as bio-scavengers. Because the particles also are chemically similar to lipoproteins, which comprise cholesterol, it might be possible to construct knedels that mimic high-density lipoproteins -- the so-called "good" cholesterol -- to scavenge low-density lipoproteins, or bad cholesterol.

Next up for Wooley is a search for the appropriate genetic material to place into the nanocage for delivery to host targets.

"We don't have a candidate yet, but we're confident we will find one," Wooley said. "We've come a long way with knedels, with still farther to go."

Wooley receives Cope scholar award

By Tony Fitzpatrick

Karen L. Wooley, Ph.D., professor of chemistry in Arts & Sciences, has been awarded the 2002 Arthur C. Cope Young Scholar Award, sponsored by the American Chemical Society (ACS).

The annual awards, this year given to two chemists age 35 or younger before April 30, 2002, are highly prestigious accomplishments in the field of organic chemistry. They are given to recognize and encourage excellence in organic chemistry.

This year's other Cope Young Scholar Award went to Matthew D. Shair, Ph.D., of Harvard University.

The award consists of $5,000, a certificate and a $40,000 unrestricted research grant to be assigned by the recipient to any university or nonprofit institution. Wooley is required to deliver a lecture at the annual Arthur C. Cope Symposium to be held as part of the ACS's annual meeting in August 2002 in Boston.

"This is wonderful news," said Joseph J.H. Ackerman, Ph.D., the William Greenleaf Elliot Professor of Chemistry and department chair. "Congratulations to Karen Wooley, and to the department. This is an extremely prestigious award that reflects well on Washington University. It's an excellent way to begin the new school year."

Wooley's award citation notes "her seminal accomplishments at the interface of organic, polymer and materials chemistry, including the development of methodologies for the preparation of well-defined nanometer-scale macromolecules, shell crosslinked knedel-like nanoparticles and nanocages, and the elucidation of original concepts in synthetic polymer chemistry for the synthesis of hydrolytically degradable polymers."

Wooley's research has drawn considerable professional and popular interest in recent years. Her work was cited in the July issue of Discover as a finalist for the annual Discover awards. She is widely considered to be in the forefront of the emerging field of nanoscopically defined organic materials.

Wooley is perhaps best known for her creation of tiny synthetic polymer particles that mimic viruses and show potential for a new direction in gene therapy and other biomedical applications. The nanoparticle goes by the descriptive name of knedel (k-ned-l), for its similarity to a popular Polish dumpling. The knedels are shell cross-linked structures surrounding a hydrophobic, or water insoluble, core domain. They are too small to be seen by the naked eye (one nanometer is one-billionth of a meter); their diameters range from 10 to 100 nanometers. They are actually close in size to many proteins and viruses.

In 2000, Wooley and researchers in her lab hollowed out the knedel core to produce "nanocages," into which someday researchers might be able to pack peptides, genes, proteins and small molecule drugs for delivery in the body.

Wooley earned a bachelor of science degree in chemistry from Oregon State University in 1988 and then studied under the direction of Jean M. J. Fr chet, Ph.D., at Cornell University, obtaining a doctorate in polymer/organic chemistry in 1993. She then began an academic career as an assistant professor of chemistry at Washington University and was promoted in 1999 to full professor with tenure.

Since 1996, Wooley also has held an appointment in the Division of Biological and Biomedical Sciences Bioorganic Chemistry Program. Her research interests include the synthesis and characterization of degradable polymers, unique macromolecular architectures and complex polymer assemblies, for which she has received young investigator awards from the National Science Foundation (1994-99), the Army Research Office (1996-99) and the Office of Naval Research (1998-2001). She was named as a DuPont Young Professor (1996-99).

Wooley currently serves the ACS Division of Polymer Chemistry as the publications chair and as an alternate councilor.

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Exhibit F

2966–2971 Nucleic Acids Research, 1999, Vol. 27, No. 14

Packaging of DNA by shell crosslinked nanoparticles

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ABSTRACT

We demonstrate compaction of DNA with nanoscale bi mimetic constructs which are robust synthetic analogs of globular proteins. These constructs are ~15 nm in diameter, shell crosslinked knedel-like (SCKs) nanoparticles, which are prepared by covalent stabilization of amphiphilic di-block co-polymer micelles, self-assembled in an aqueous solution. This synthetic approach yields size-controlled nanoparticles of persistent shape and containing positively charged functional groups at and near the particle surface. Such properties allow SCKs to bind with DNA through electrostatic interactions and facilitate reduction of the DNA hydrodynamic diameter through reversible compaction. Compaction of DNA by SCKs was evident in dynamic light scattering experiments and was directly observed by *In situ* atomic force microscopy. Moreover, enzymatic digestion of the DNA plasmid (pBR322, 4361 bp) by *Eco*RI was inhibited at low SCK:DNA ratios and prevented when ≤60 DNA bp were bound per SCK. Digestion by *Msp*I in the presence of SCKs resulted in longer DNA fragments, indicating that not all enzyme cleavage sites were accessible within the DNA/SCK aggregates. These results have implications for the development of vehicles for successful gene therapy applications.

INTRODUCTION

Biomimicry (1,2), which relies upon guidance from biology and biotechnology in the design of novel synthetic or natural materials, is emerging as an effective strategy in diminishing the size of devices while maintaining their complex functionality. Since the size of many functional biological structures falls in the 10–100 nm range, it is appropriate to seek methods for preparation of functional analogs within the newly developing field of nanotechnology. Herein, we present a novel approach for mimicking the basic features of globular proteins, such as their overall size, shape and surface charge. This approach relies on self-assembly of amphiphilic synthetic di-block copolymers into globular core–shell nanostructures, followed by stabilization via covalent crosslinking. Since crosslinking occurs selectively within the shell of assembled polymer micelles, these novel constructs are referred to as shell crosslinked knedel-like (SCK) nanospheres. (*Knedel* is a polish term for dumplings, having a spherical shape and core–shell morphology. For synthetic nanomaterials of similar morphology see 3.) Owing to their amphiphilic core–shell morphology, SCKs belong to the same category as globular amphiphilic biological nanostructures such as lipoproteins, viruses, globular proteins, etc., in broad physicochemical terms. These synthetic constructs could prove to be important for biomedical applications, particularly those involving gene delivery and expression.

Handling of long DNA chains within the limited space available in cells is facilitated by various compaction mechanisms (4–6). Several synthetic systems, including liposomes (7–9), linear polymers (10,11), cationic lipid–peptoid conjugates (12), polymer micelles (13,14), dendrimers (15) and organic nanoparticles (16–18) have been shown to bind DNA and provide protection against enzymatic digestion. The non-particulate nature of liposomes and linear polymers apparently results in the complexation of DNA through non-specific electrostatic interactions leading to a heterogeneous binding character (8,9,11). Polymer micelles typically exist with diameters of 10–100 nm and they possess a core–shell morphology that can contain cationic surface charges, however, the micellar organization is a self-assembled structure that can be easily deformed or destroyed during complexation with DNA due to the absence of covalent stabilization. Other known globular macromolecular entities, such as dendrimers, are covalently bound macromolecules, but they are not readily synthesized to 10 nm diameters (19,20). Previously studied organic nanoparticles were typically >>50 nm with broad size distributions. Herein, we report the interaction of DNA with stable, synthetic SCKs (shown schematically in Fig. 1) of ~15 nm diameter (3), which is intermediate between the sizes available for dendrimers and organic nanoparticles. We also illustrate the effect of compaction on the accessibility of DNA toward the action of digestive enzymes and show evidence of DNA release from the DNA/nanoparticle complexes.

MATERIALS AND METHODS

Materials

DNA plasmid pBR322, *Eco*RI, *Msp*I, NEBuffer 1, NEBuffer 2, pBR322 DNA *Msp*I digest and Lambda DNA *Bst*EII digest were purchased from New England BioLabs (Beverly, MA) and used as received. Tris base (Sigma), sodium acetate

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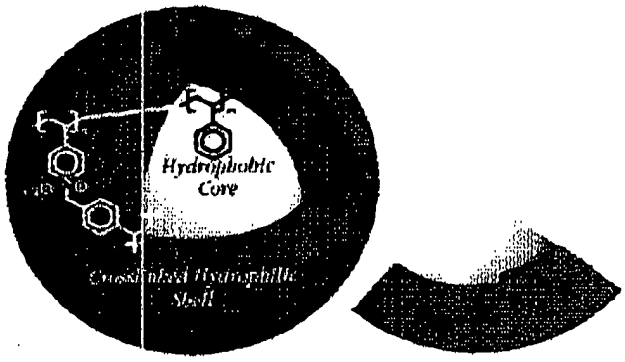

Figure 1. Schematic representation of the shell crosslinked knedel-like (SCK) nanosphere, containing a crosslinked, positively charged, hydrophilic shell layer surrounding the hydrophobic core domain. The SCKs are prepared from assembly of block co-polymers of polystyrene and *p*-chloromethylstyrene-quaternized poly(4-vinyl pyridine) into polymer micelles, followed by crosslinking through reaction of the styrenyl moieties located within the shell.

(Sigma), EDTA dihydrate (Sigma) and agarose (Fisher) were used as received. The experimental details for the preparation of the SCK nanospheres are described elsewhere (21). The SCK composition consisted of a polystyrene (PS) core encapsulated within a layer of poly(4-vinyl pyridine) (PVP) that had been quaternized with 4-chloromethylstyrene to provide crosslinking throughout the shell layer. The ratio PS:PVP was 1:2, with 46% of the pyridyl nitrogens quaternized by reaction with 4-chloromethylstyrene (21).

General procedure for the cleavage of pBR322 with *Eco*RI in the presence of SCK

Complexes A–C were prepared by mixing 1 µl pBR322 (1 µg/µl), 1, 3 or 5 µl SCKs (8×10^{-6} M) and 1 µl HEPES (1 M). Total volumes were raised to 10 µl with doubly distilled water if the restriction enzyme was not added. If restriction enzyme was to be added, the total volume was raised to 8.8 µl with doubly distilled water and 1 µl 10× NEBuffer 1 and 0.2 µl *Eco*RI (20 000 U/ml) were added. The samples were incubated at 37°C for 3 h, vacuum centrifuged for ~5 min and 6 µl of 2× gel loading buffer were added. Samples were analyzed by electrophoresis at 55–65 V for 2–2.5 h with a 1% agarose gel in 1× acetate buffer. DNA was visualized with ethidium bromide (1 µg/ml of gel).

General procedure for timed digestion experiments

Complexes A–C were prepared at four times the volume by mixing 4 µl pBR322 (1 µg/µl) with 4, 12 or 20 µl SCKs (8×10^{-6} M). Total volumes were raised to 35.2 µl with doubly distilled water. An aliquot of 4 µl of 10× NEBuffer 1 was added to each sample, followed by addition of 0.8 µl *Eco*RI (20 000 U/ml). The samples were incubated at 37°C with aliquots removed at the specified intervals. After removal of the last aliquot, 6 µl of 2× loading buffer were added to each sample. Samples were analyzed by electrophoresis at 55–65 V for 2–2.5 h with a 1% agarose gel in 1× acetate buffer. DNA was visualized with ethidium bromide (1 µg/ml of gel). Controls were run on the same gel under the same conditions. Spot densitometry of the DNA on the gel was made using the NIH shareware tnimage (http://las1.ninds.nih.gov/pub/unix/).

General procedure for the cleavage of pBR322 with *Msp*I in the presence of SCK

Formation of complex A has been described. Complex D was prepared by mixing 1 µl pBR322 (1 µg/µl), 0.5 µl SCK (8×10^{-6} M) and 1 µl HEPES (1 M). Total volumes were raised to 10 µl with doubly distilled water if the restriction enzyme was not added. If restriction enzyme was to be added, 1 µl of 10× NEBuffer 2 was added to each sample, followed by addition of 0.8 µl *Msp*I (20 000 U/ml). The samples were incubated at 37°C for 3 h, vacuum centrifuged for ~5 min and 6 µl of 2× gel loading buffer were added. Marker solutions consisted of 1.5 µl of marker and 6 µl of 2× gel loading buffer. Samples were analyzed by electrophoresis at 55–65 V for 2 2.5 h with a 1% agarose gel in 1× acetate buffer. DNA was visualized with ethidium bromide (1 µg/ml of gel).

Measurement of melting curve of DNA and SCK/DNA complexes

Melting transitions were measured upon aqueous solutions of DNA and an SCK/DNA complex with a molar ratio of 80:1. DNA melting curves were obtained in a Cary 1e at 260 nm with a heating rate of 5°C/15 min (the temperature was raised 5°C and allowed to equilibrate for 15 min) from 50 to 90°C.

Atomic force microscopy (AFM) of SCK/DNA complexes

All AFM observations were carried out with the aid of a Nanoscope III-M system (Digital Instruments, Santa Barbara, CA) equipped with a vertical engage J-scanner. The imaging conditions were as follows: TESP tapping mode silicon cantilevers (nominal spring constant 50 N/m, typical resonance frequency in the range 250–300 kHz); cantilever oscillation amplitude 0.5 V (non-calibrated signal); set-point corresponding to 95% of free oscillation amplitude; scan frequency 3 Hz; integral gain 0.7, proportional gain 5.0 (instrument settings in arbitrary units).

The sample was prepared by placing a 2 µl drop of solution containing DNA (10 µg/ml, 4.6 kb circular luciferase expression vector) and SCKs in 10 mM HEPES, 2 mM $MgCl_2$, pH 7.6, on a surface of freshly cleaved mica (New York Mica Company, New York, NY); after ~5 s, necessary for binding of the DNA and SCKs to the mica, the excess solution was washed away with 200 µl of ultrapure water and the surface was dried under a stream of nitrogen.

Tapping mode imaging under buffer was performed using a standard contact mode fluid cell and 100 µm, wide legged, silicon nitride cantilevers (nominal spring constant 0.58 N/m). The cantilever was oscillated by applying a sinusoidal voltage across the Z-direction of the scanner (22), which required minor modification of the controller electronics, performed according to the manufacturer's instructions. Best results were obtained when the cantilever was driven at a frequency range corresponding to the broad maximum of cantilever oscillation amplitude centered around 8–9 kHz (23–25). Imaging conditions were as follows: cantilever oscillation amplitude signal (uncalibrated) 0.5–1.0 V; set-point corresponding to above 90% of free oscillation amplitude; integral and proportional gains 0.3 and 2.0 (instrument settings in arbitrary units); scan frequency 1–4 Hz.

Samples for imaging under liquid were prepared in a 10 mM HEPES, 1.5 mM $ZnCl_2$, pH 7.6, buffer. A 30–40 µl drop of SCK–DNA solution was placed directly on the working surface of a fluid cell, which was then positioned on the scanner. Imaging was carried out after several minutes, which was necessary for equilibration of the solution in contact with the mica.

Dynamic light scattering (DLS) of SCK/DNA complexes

The DLS instrumentation was a Brookhaven Instruments Co. (Holtsville, NY) system containing a model BI-200SM goniometer, a model EMI-9865 photomultiplier and a model BI-9000AT digital correlator. Incident light was provided by a model 95-2 Ar ion laser (Lexel Corp., Palo Alto, CA) operated at 514.5 nm. All measurements were made at 20°C. Prior to analysis, solutions were centrifuged in a model 5414 microfuge (Brinkman Instrument Co., Westbury, NY) for 6 min to sediment dust.

Scattered light was collected at a fixed angle of 90°. The digital correlator was operated with 200 channels, a dual sampling time of 100 ns, a 5 µs ratio channel spacing and a duration of 5 min. A photomultiplier aperture of 200 µm was used and the incident laser intensity was adjusted to obtain a photon counting rate of 83 000 c.p.s. Only measurements in which measured and calculated baselines of the intensity autocorrelation function agreed to within 0.1% were used to calculate particle size. The calculation of particle size distribution and distribution averages was performed with the ISDA software package (Brookhaven Instruments Co., Holtsville, NY) which employed single exponential fitting, cumulants analysis and non-negatively constrained least squares particle size distribution analysis routines (26).

RESULTS AND DISCUSSION

SCKs, which are essentially stabilized polymer micelles, are constructed by a combination of covalent and non-covalent interactions to readily build up spherical polymer nanoparticles of the appropriate size and chemical composition for interaction with DNA. The SCKs are prepared in a three-step process, including: (i) the synthesis of a linear amphiphilic block copolymer; (ii) self-assembly into spherical micelles in aqueous solution; (iii) stabilization by intramicellar crosslinking of functionalities located within the shell domain. The SCKs used in this study consisted of hydrophobic PS cores and hydrophilic, crosslinked *p*-chloromethylstyrene-quaternized PVP shells (21). SCKs prepared from polymer chains of 20 700 Da molecular weight and a ratio of PS:PVP repeat units of 1:2 have a solid-state diameter of 9 ± 3 nm and a hydrodynamic diameter of 16 ± 2 nm, determined by AFM and DLS, respectively. The positive charges, an average of 500 per SCK, are located throughout the several nanometer thick shell, so that all cations are not expected to be accessible for strong interaction with the DNA phosphate ester anions.

The aggregation of DNA with SCKs was studied by a combination of AFM (27,28), DLS and enzymatic digestion experiments. Initial evidence of adhesive interaction between SCKs and plasmid DNA was obtained from AFM observations of dehydrated samples adsorbed on mica, which revealed the presence of bulky aggregates of the SCKs with partially exposed segments of unbound DNA (Fig. 2). These aggregates markedly differed from aggregates of SCKs on mica in the absence of DNA. Typical morphologies of aggregates of free SCKs, depending on concentration of starting solution, ranged from scattered single particles (26) through monolayer patches (3) to uniform films (data not shown). It is important to note that SCKs in complexes with DNA maintained their original size and shape. However, the supercoiled form of the DNA appears to have been perturbed in some regions. Further confirmation of the supercoiling perturbation of plasmid complexed with SCKs was obtained from the UV/vis spectroscopic studies of DNA melting; thermal denaturation of the plasmid alone occurred at 75°C, whereas the melting temperature of the DNA was lowered to 70°C in Complex B.

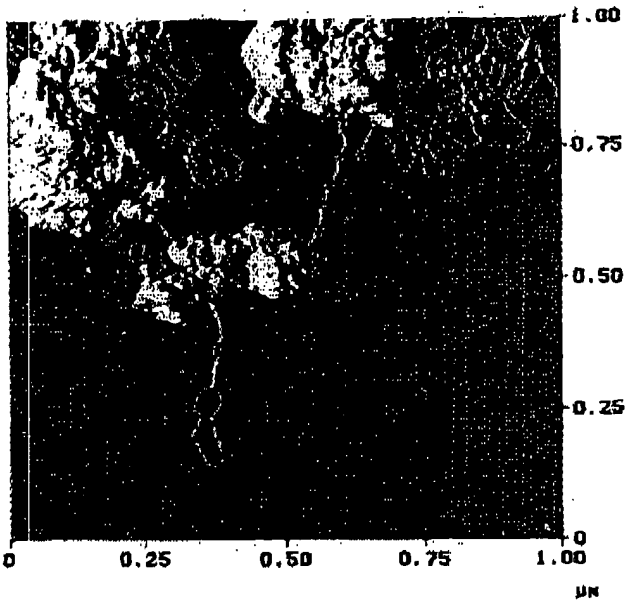

Figure 2. Tapping mode AFM image of DNA/SCK aggregates dried on the surface of mica, demonstrating SCK/DNA binding with maintenance of the SCK size and shape. Some regions exhibit unbound DNA, while others display SCKs bound along the DNA in a similar fashion as previously observed in AFM images of chromatin (31).

Further insights into the mechanism of compaction were obtained from *in situ* AFM experiments carried out under solution with increasing ratios of SCK:DNA plasmid (pBR322, 4361 bp). As illustrated in Figure 3A, at a SCK:DNA molar ratio of 20:1 (as in Complex A) the majority of DNA was still in the unbound supercoiled state. This can be understood by bearing in mind that despite the large molar excess of SCKs at this stoichiometry, the charge ratio between SCK and DNA plasmid just reaches the value of 1:1. (The ratio of charges is determined from the *p*-chloromethylstyrene quaternization extent of the PVP SCK shell material, which gives cationic sites, and assuming two negative charges per base pair.) At higher SCK:DNA molar ratios, more compact complexes, such as those shown in Figure 3B (globular aggregate with some DNA loops still visible outside) and in Figure 3C (globular complex with no DNA visible) were observed.

DLS measurements (29) confirmed that the SCK/DNA aggregates formed while in solution and provided for

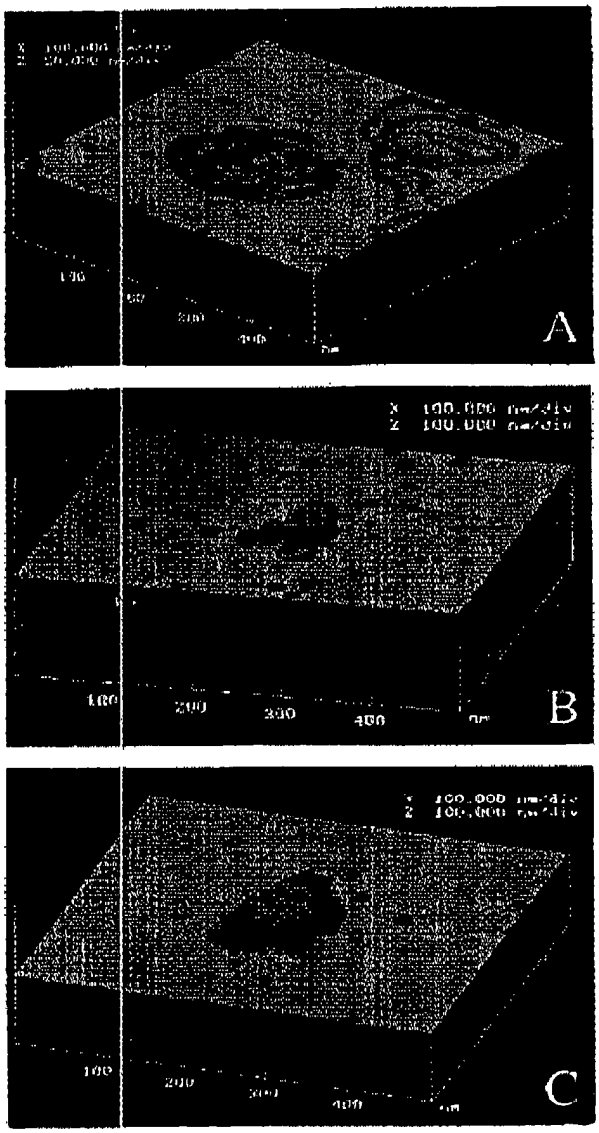

Figure 3. *In situ* tapping mode AFM images of aggregates of SCKs with DNA (pBR322, 4361 bp) adsorbed on freshly cleaved mica and visualized under buffer (10 mM HEPES, 1.5 mM $ZnCl_2$, pH 7.6). (**A**) SCK bound to the plasmid DNA (2 nM SCK, 0.1 nM DNA, molar ratios as in complex A; see Table 1); (**B**) a globular aggregate with loops of DNA still visible (8 nM SCK, 0.1 nM DNA); (**C**) large SCK/DNA aggregate with no identifiable DNA (8 nM SCK, 0.1 nM DNA). The presence of Zn^{2+} in the buffer used for *in situ* imaging was necessary to facilitate attachment of DNA/SCK complexes to mica (32). In control experiments with DNA alone, in which weaker binding Mg^{2+} was used, DNA was observed to change position on the surface from scan to scan. Under similar conditions, complexes of SCKs with DNA were bound to the surface more weakly than DNA alone and their bulky character made them more susceptible to displacement by the AFM tip.

determination of the average diameter values for the aggregates (Table 1). The mean volume-weighted hydrodynamic diameters (D_m) increased by at least a factor of four ($D_{m,i}/D_{m,SCK}$) for each of the complexes in comparison to SCK, suggesting

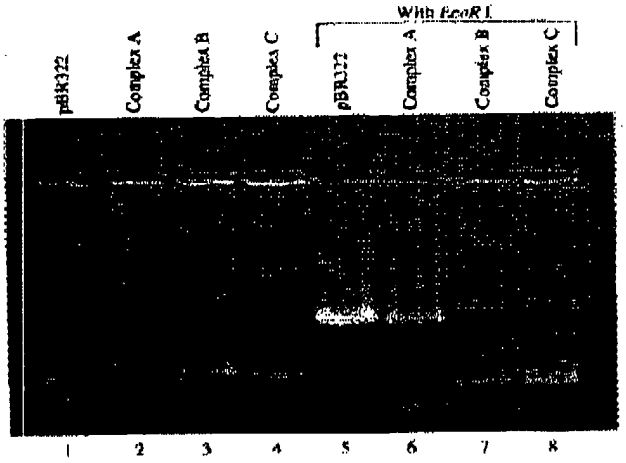

Figure 4. Complexation of pBR322 with SCK offers protection from *Eco*RI digestion. The DNA and DNA/SCK complexes (A–C) were incubated for 3 h at 37°C in the absence (lanes 1–4) and presence (lanes 5–8) of *Eco*RI.

that the interactions between DNA and SCK produce complexes that are multimeric in SCK.

The hydrodynamic diameter distribution of the DNA used to prepare SCK/DNA complexes was also characterized by DLS. Unlike the SCK component of the complexes, the diameter distribution of the DNA component was extremely broad. The broad size range of DNA molecules apparent in the DLS results is consistent with the heterogeneity found in electrophoresis gels of the DNA; the plasmid and its cleaved forms are observed and each is expected to adopt many conformations that are non-spherical and that can lead to a continuous size distribution rather than discrete entities. Diameters ranged from 15 to >3000 nm, with a calculated intensity-weighted mean diameter of 562 nm. In contrast, diameters of all the SCK/DNA complexes were <300 nm (Table 1) and of lower size dispersity.

The accessibility of the DNA within the SCK/DNA complexes was evaluated by enzyme digestion experiments, using a restriction endonuclease, *Eco*RI, and was found to be dependent on the extent of aggregation. As illustrated in Figure 4, the DNA/SCK complexes are destroyed during gel electrophoresis (lanes 2–4) and unbound DNA is observed, which provides a method for analysis of DNA cleavage under the action of enzymes. It is important to note that this release of the DNA, which is a prerequisite for gene therapy applications, is unique to SCKs. Each of the previously studied systems listed above have shown binding to DNA, but no apparent release of the DNA, observed as reduced DNA mobility upon gel electrophoresis. Upon incubation in the presence of *Eco*RI for 3 h at 37°C, the unbound DNA and the loosely bound DNA/SCK complex (Complex A) allowed for complete cleavage of the supercoiled plasmid to an open linear form (lanes 5 and 6, respectively), whereas Complexes B and C offered protection to the DNA (lanes 7 and 8, respectively).

Timed digestion experiments revealed that while all SCK/DNA complexes inhibited DNA cleavage, inhibition time increased with an increase in the molar ratio of SCK:DNA.

Table 1. SCK/DNA complexes studied and the hydrodynamic diameter distribution data from dynamic light scattering[a]

Complex	Molar ratio (SCK:DNA)	Charge ratio (SCK:DNA)	Base pairs per SCK	D_1[b] (nm)	V_1 (vol%)	D_2[b] (nm)	V_2 (vol%)	D_m[c] (nm)	$D_{m,i}/D_{m,SCK}$[d]
A	20	1:1	200	36	80	280 + 170	20	85	4.9
B	70	4:1	60	120	>99	<5	<1	120	6.7
C	120	7:1	40	26	85	120 + 32	15	40	2.4
SCK alone	-	-	0	15	99	>100	1	16	1.0

[a]All measurements were made at 20°C and reported hydrodynamic diameters and volume % are mean values of two determinations.
[b]Diameter distributions were calculated from autocorrelation functions of light scattering intensities using a multiple pass non-negatively constrained least squares fitting (NNLS) algorithm in the ISDA software package from Brookhaven Instruments Co. (Holtsville, NY). A bimodal distribution of particles was observed, with diameters D_1 and D_2 for volume fractions V_1 and V_2.
[c]D_m is the volume-averaged mean particle or aggregate diameter, calculated as $D_m = (D_1 \cdot V_1 + D_2 \cdot V_2)/(V_1 + V_2)$.
[d]$D_{m,i}/D_{m,SCK}$ is the ratio of D_m values for the complexes versus the D_m value of the SCK, where i = A, B or C.

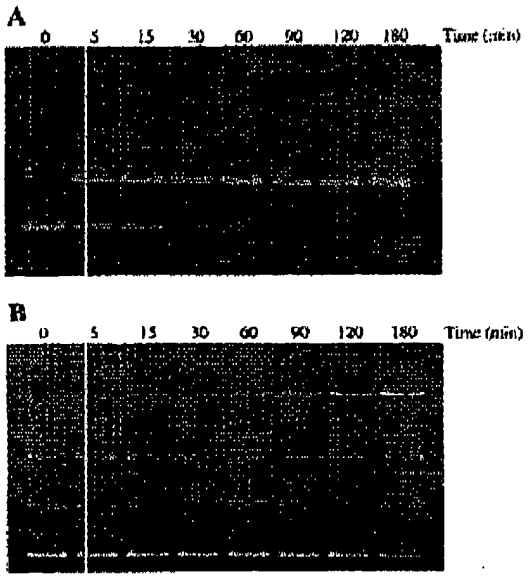

Figure 5. Cleavage of pBR322 by *Eco*RI is inhibited at low SCK content and is prevented at high SCK content. (**A**) Complex A incubated with *Eco*RI for 0–180 min. Cleavage of all DNA occurred by 60 min. (**B**) Complex C incubated with *Eco*RI for 0–180 min. No measurable cleavage products are observed.

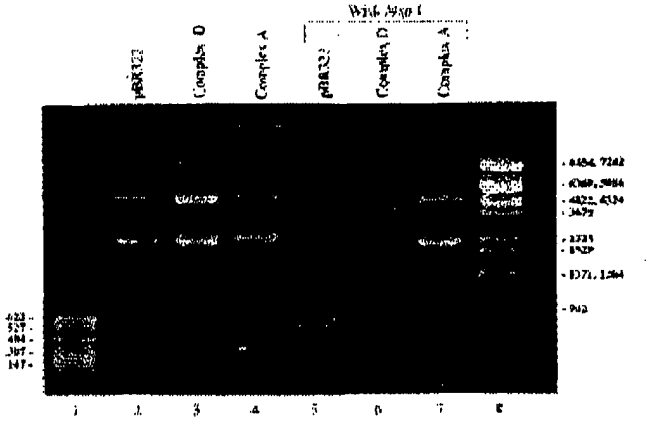

Figure 6. A portion of sites are not accessible to enzymatic digestion when the digestion is performed with *Msp*I (a restriction endonuclease that cleaves pBR322 at 26 sites) upon pBR322 in the presence of SCKs. Even with a 180 min incubation time, cleavage is prevented for Complex A (lane 7). Cleavage of the DNA of Complex D (SCK:DNA molar ratio 12:1, charge ration 0.7:1) results in less extensively fragmented DNA (lane 6) than found for *Msp*I digestion of pBR322 without the SCK nanoparticles present (lane 5). Lane 1 is the marker pBR322 DNA *Msp*I digest and lane 8 is the marker Lambda DNA *BstE*II digest.

Digestion was entirely prevented at the SCK:DNA molar ratio of 70:1 (60 bp per SCK). Representative gels for the digestion experiments with low and high SCK content are shown in Figure 5. For Complex A, the disappearance of the supercoiled DNA plasmid corresponds to the appearance of the cleaved DNA (Fig. 5A). In contrast, for Complex C (Fig. 5B), disappearance of supercoiled DNA does not lead to an increase in the amount of cleaved DNA, instead, at longer incubation times extensive aggregation of residual material in the well is observed.

Further evidence for protection of SCK-compacted DNA was obtained with *Msp*I, a restriction endonuclease that cleaves pBR322 at 26 sites. At SCK:DNA ratios that provide for inhibition but still allow for partial DNA digestion, the lengths of the majority of DNA cleavage products were slightly larger than 702, 1371 and ~3675 bp (Fig. 6, lane 6), whereas unprotected DNA was cleaved into lengths of ≤622 bp (Fig. 6, lane 5). The increased lengths of the degradation products is expected to result from interaction of the DNA with the SCKs, thus limiting the number of sites that are accessible to the enzyme, and is perhaps an indication of a selective binding mode.

We have demonstrated that synthetic functional nanostructures that are designed to exhibit just the basic physicochemical attributes (size, shape persistence and surface charge) of proteins involved in DNA compaction can effectively compact DNA and protect it from enzymatic digestion.

Importantly, these novel constructs maintain their size and shape upon complexation and they are also capable of releasing the DNA, in analogy with biological systems. The observed protection of SCK-compacted DNA can be explained as an effect of enclosure of DNA within the bulk of aggregates. Experiments with short, linear DNA fragments are expected to reveal whether SCKs are capable of binding and protecting DNA by a mechanism that involves selective binding at a single particle level, which would make them even closer mimics of DNA compacting proteins such as histones. SCKs show promise as synthetic nanoparticulate compacting agents for control of DNA accessibility and may find applications in gene transfection and expression. They offer the additional unique advantages of a well-defined structure and modifiable surface chemistry (3) to target site-specific delivery routes.

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